Development and Application of a Simple Microassay for Adenosine in Rat Plasma

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SUMMARY Adenosine may be a physiological modulator of vascular smooth muscle tone, sympathetic neurotransmission, renin release, and renal and cardiac function. To facilitate the elucidation of the physiological role of adenosine, a microassay for adenosine was developed that allows accurate quantitation of adenosine in 75 μl of rat plasma, thus permitting multiple determinations of plasma adenosine levels in an individual rat without inducing hemodynamic perturbations due to blood loss. The technique employs a simple and rapid extraction of plasma with a reverse-phase Sep-Pak cartridge and exploits the increased mass sensitivity of microbore high performance liquid chromatography. The assay was verified by demonstrating 1) a linear relationship between the amount of adenosine added to plasma and the amount detected by the assay, 2) a linear relationship between the rate of adenosine infusion into rats and plasma adenosine levels, and 3) the absence of measurable adenosine levels in plasma incubated with adenosine deaminase. The mean arterial plasma level of adenosine in the anesthetized rat was determined to be 119 ± 28 (SD) ng/ml (n = 10). With the use of this assay, renal venous plasma levels of adenosine were found to be elevated sixfold in two-kidney, one clip Goldblatt hypertensive rats (1 week postclipping) compared with sham-operated controls. Given the known effects of adenosine on renin release, these data support a role for endogenous adenosine as a regulator of renin release in renovascular hypertension. (Hypertension 10: 189-197, 1987)

KEY WORDS • adenosine • renovascular hypertension

INTEREST in adenosine as a possible mediator or modulator of physiological processes has been sustained for many years. Adenosine has been implicated as a mediator of reactive hyperemia,1 reactive ischemia,1 regulation of the splanchnic circulation,4 tubuloglomerular feedback,2 and renovascular hypertension.8 In addition, adenosine has been implicated as a modulator of noradrenergic neurotransmission9 and renin release.8 Even though considerable attention has been focused on the possible role of adenosine in physiology and pathophysiology, little is actually known at present about the import of adenosine in vivo. This paucity of information has been due to the unavailability of specific adenosine receptor antagonists and the lack of adequate techniques for quantitating the level of endogenous adenosine in interstitial fluid. Although recent advances in the medicinal chemistry of xanthine derivatives may have solved the first problem and may provide the physiologist with specific and potent adenosine receptor antagonists,9 the problems associated with quantitating changes in interstitial adenosine levels still remain. One technique of assessing changes in interstitial adenosine levels has been to freeze-clamp organs and measure total tissue content of adenosine. The major problems with this approach are that multiplex sampling from undisturbed organs is not possible and measurements include both intracellular and extracellular adenosine. Since the physiologically relevant adenosine receptors are extracellular, total tissue adenosine levels are difficult to interpret.

Another approach used to assess interstitial adenosine levels is to measure adenosine in venous plasma from the organ of interest. This method allows convenient access, permits repeated determinations of adenosine levels without disturbing the organ, and provides an estimate of changes in interstitial adenosine levels, assuming a constant relationship between interstitial and plasma adenosine concentrations.
Several assays for plasma adenosine using conventional high performance liquid chromatography (HPLC) and ultraviolet (UV) detection have been published. However, the sensitivity of previously published assays does not permit multiple sampling in small laboratory rodents without inducing unacceptable blood loss. Although a procedure for measuring adenosine with the prerequisite sensitivity has been published, this procedure requires derivatization, entailing additional preparative steps, and the applicability of this assay to plasma has not yet been established.

In the last few years, new methods for increasing the mass sensitivity of conventional HPLC systems linked to absorbance detection have been invented. In particular, mass sensitivity has been greatly improved (approximately 10- to 30-fold) by the recent advent of microbore HPLC systems, in which extracolumn variance is reduced to vanishingly small levels by directly interfacing a low volume injector, column, and flow cell. In this report, we describe a new assay for adenosine in which the enhanced mass sensitivity of microbore HPLC is exploited to accurately determine adenosine levels in 75 μl of rat plasma. The assay is simple, rapid, and inexpensive and provides a sensitivity permitting serial determinations of plasma adenosine levels in an individual rat.

Recently, we discovered that caffeine markedly exacerbates two-kidney, one clip (2K1C) renovascular hypertension in rats. Associated with the elevated blood pressure was a sevenfold increase in plasma renin activity in hypertensive rats treated with caffeine as compared with untreated hypertensive rats. Since caffeine is an adenosine antagonist, and since adenosine is a potent inhibitor of the renin release response to renal artery hypotension (unpublished observation 1986), we hypothesized that in the renovascular hypertensive rat adenosine may restrain the renin release response to renal ischemia. Interruption of this restraint by caffeine might lead to a greater renin release and, therefore, is sufficiently lipophilic to be retained in the plasma during the processing required only a few seconds.

Sample Preparation

Immediately after collection, the blood sample was centrifuged at 2000 g for 10 minutes at 4°C. After centrifugation, exactly 200 μl of supernatant fluid (containing approximately 75 μl of plasma and 125 μl of stopping solution, depending on the hematocrit (Hct)) was withdrawn and placed in a 12 x 75-mm polypropylene tube. Samples showing any visible hemolysis were discarded. Next, 100 ng of N\(^{-}\)-dimethylguanosine (internal standard, 10 μl) dissolved in water was added to the 200 μl of supernatant fluid, and the supernatant fluid was agitated. The samples were deproteinized without delay by adding 60 μl of 15% trichloroacetic acid. The precipitated proteins were removed by centrifugation at 2000 g for 10 minutes, and the supernatant fluid was removed and extracted immediately over a reverse-phase Sep-Pak cartridge. (Waters Associates, Milford, MA, USA).

Surprisingly, it was not necessary to neutralize the supernatant fluid before application to the reverse-phase Sep-Pak cartridge provided proteins were precipitated with trichloroacetic acid. In contrast, if proteins were precipitated with perchloric acid, adenosine was not retained by a reverse-phase Sep-Pak cartridge unless the sample was first neutralized. One interpretation of these observations is that, although adenosine is ionized at low pH, in the presence of excess trichloroacetic acid it exists as an ion pair with trichloroacetate and, therefore, is sufficiently lipophilic to be retained by a reverse-phase Sep-Pak cartridge.

Before extraction, a C\(_{18}\) Sep-Pak cartridge was attached to the barrel of a 3-ml plastic disposable syringe and placed in a rack providing vertical support. The Sep-Pak cartridge was activated with 2 ml of methanol and washed with 2 ml of water. Next, the 200 μl of supernatant fluid was applied to the Sep-Pak cartridge with a disposable glass pipet, and the sides of the 3-ml syringe were washed with 200 μl of water. With the use of the syringe plunger, the sample in the 3-ml syringe was loaded onto the Sep-Pak cartridge. The Sep-Pak cartridge was washed with 2 ml of water and then with 2 ml of methanol/water (5:95). This was done by loading the 3-ml syringe with the appropriate solution and forcing the fluid through the Sep-Pak cartridge with positive pressure (i.e., syringe plunger). The adenosine and internal standard were then eluted from the column with 2 ml of methanol/water (50:50) and collected into a 12 x 75-mm borosilicate tube. The eluent was then poured into a second 3-ml plastic disposable syringe equipped with a 0.45-μm filter (Milli-
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The amount of adenosine in a plasma sample was calculated by measuring the peak height ratio of adenosine to internal standard and applying this ratio in the regression equation. Since the original blood sample was diluted 1:1 with stopping solution, the 200 µl of supernatant fluid to which internal standard was added contained less than 50% plasma. The amount of plasma assayed was calculated from the equation: plasma volume assayed = (200 µl)(1 - Hct/2 - Hct). For a normal Hct of 40%, the assayed plasma volume was 75 µl. The concentration of adenosine in plasma was then calculated by dividing the amount of adenosine in the sample by the actual volume of plasma assayed.

Assay Verification

Step 1: Linearity of Standard Curve

A standard curve was prepared by adding 0, 10, 20, 40, 60, 80, or 100 ng of adenosine and 100 ng of N²-dimethylguanosine to 10 µl of mobile phase. The peak height ratios were determined as described and plotted as a function of amount of adenosine per 100 ng of internal standard. The same standard curve was analyzed on two occasions separated by 4 weeks to determine the stability of the standard curve at 4°C and the reproducibility of peak height ratios with the Isco microLC system over time.

Step 2: Recovery of Adenosine and N²-Dimethylguanosine from Extraction, Filtration, and Sample Volume Reduction Procedures

A normal 16-week-old Sprague-Dawley rat was anesthetized with pentobarbital (50 mg/kg i.p.), and the left carotid artery was cannulated with polyethylene tubing (PE-50). A 2 ml blood sample was drawn into 2 ml of stopping solution, and the sample was centrifuged at 4°C. The supernatant fluid from this sample was divided into six 200-µl portions, and 10, 40, 60, 80, or 100 ng of adenosine was added to five of the 200-µl samples; the sixth sample did not receive adenosine. The five samples spiked with adenosine also received 100 ng of N²-dimethylguanosine. All six samples were processed as described (see Sample Analysis section), and the amount of adenosine and N²-dimethylguanosine in each sample was determined by comparing sample peak heights with a standard curve for adenosine or N²-dimethylguanosine. The amount of endogenous adenosine, determined from the sixth sample, was subtracted from the amount of adenosine measured in Samples 1 through 5 to give the amount of exogenous adenosine that was recovered. The percentage recoveries of adenosine and N²-dimethylguanosine were calculated.

Step 3: Relationship Between Amount of Adenosine Added to Supernatant Fluid and Amount Detected by Assay

The purpose of Step 3 was to determine whether the assay afforded a linear response with a slope of 1 to adenosine present in samples. Blood (2 ml) from a normal Sprague-Dawley rat was collected into 2 ml of stopping solution as described in Step 2. Supernatant fluid from this sample was divided into seven portions, and each portion was spiked with adenosine (0, 10, 20, 40, 60, 80, or 100 ng) and N²-dimethylguanosine (100 ng). Samples were processed as described above (see...
Sample Analysis section), and the amount of adenosine detected by assay was graphed as a function of adenosine added to the supernatant fluid.

**Step 4: Stability of Adenosine in Blood Sample Collected into Stopping Solution**

Adenosine is rapidly taken up into cells and is degraded by adenosine deaminase. The purpose of the stopping solution was to halt adenosine uptake by formed elements in blood (dipyridamole) and to inhibit degradation of adenosine by adenosine deaminase (EHNA). As a normal routine, samples were always centrifuged immediately after collection and deproteinized as quickly as possible. However, concern remained that adenosine might disappear from the sample during the steps between collection and deproteinization. Further, for logistic reasons, an experimenter may not always be able to centrifuge and deproteinize each sample as soon as it is collected. Therefore, the effect of delaying sample processing on adenosine levels had to be determined. To determine the extent to which adenosine levels declined after sample collection, a blood sample (3 ml) from a Sprague-Dawley rat was collected into stopping solution (3 ml) as described in Step 2. The sample was divided into two portions, one portion was centrifuged immediately, and the supernatant fluid was assayed in quadruplicate. The second portion of blood remained on ice for 2 hours and then was centrifuged and assayed in quadruplicate.

**Step 5: Coefficient of Variation**

A 2-ml blood sample from a Sprague-Dawley rat was collected into 2 ml of stopping solution as described in Step 2. The sample was centrifuged, and six 200-μl aliquots of the supernatant fluid were assayed as described (see Sample Analysis section).

**Step 6: Adenosine in Sample Treated with Adenosine Deaminase**

Two blood samples (200 μl each) were withdrawn sequentially from a donor rat. One sample was collected into stopping solution (as in Step 2) and assayed immediately. The other sample was incubated for 20 minutes at 37 °C (water bath) with 5 units of adenosine deaminase and then processed normally (see Sample Analysis section).

**Step 7: Effect of Adenosine Infusion on Plasma Adenosine Level**

Three male Sprague-Dawley rats were anesthetized with pentobarbital (50 mg/kg i.p.), and the jugular vein and thoracic aorta (through the left carotid artery) were cannulated with polyethylene tubing (PE-50). The aortic cannula was connected to a Hewlett-Packard (Palo Alto, CA, USA) pressure transducer, and blood pressure was recorded on a Hewlett-Packard recorder (Model 7768A). During the control period, 0.9% saline was infused into the jugular catheter at a rate of 50 μl/min. A baseline blood sample for plasma adenosine was withdrawn from the aorta, and then adenosine dissolved in 0.9% saline was infused into the jugular vein at 0.016 mg/min (50 μl/min) in one rat and 0.031 mg/min in two other rats. Twenty minutes into the infusion, a second arterial blood sample was withdrawn. This procedure was repeated for two additional doses of adenosine (0.062 and 0.25 mg/min). All blood samples were analyzed for adenosine as described (see Sample Analysis section).

**Step 8: Adenosine Plasma Level in Normal Rats**

Ten rats were anesthetized withpentobarbital (50 mg/kg i.p.), and blood was withdrawn from the aorta and analyzed as described (see Sample Analysis section).

**Renal Venous Plasma Levels of Adenosine in Sham-Operated and 2K1C Goldblatt Hypertensive Rats**

Male Sprague-Dawley rats (weight, 200–220 g) were anesthetized withpentobarbital (50 mg/kg i.p.), and a silver clip (0.25-mm gap) was placed on the left renal artery. In six of the rats the clip was left in place (2K1C), and in the other six the clip was removed (sham-operated rats). The abdomen was sutured closed, and the skin was closed with wound clips. One week later, each animal was reanesthetized withpentobarbital and a 200-μl blood sample was removed from the right and left renal veins. Blood samples were collected, extracted, and analyzed as described.

**Chemicals**

Chemicals were obtained from the following sources: adenosine and dipyridamole (Sigma Chemical Corp., St. Louis, MO, USA); EHNA (Wellcome Research Laboratories, Research Triangle Park, NC, USA); N²-dimethylguanosine (Pharmacia P-L Biochemicals, Piscataway, NJ, USA); water, acetonitrile, H₃PO₄, trichloroacetic acid, and methanol (Fisher, Fair Lawn, NJ, USA). All solvents were HPLC grade.

**Results**

**Assay Verification**

**Step 1: Linearity of Standard Curve**

Figure 1 depicts two standard curves for adenosine in HPLC mobile phase that were obtained from the same solutions, but on two occasions separated by 4 weeks. These data indicate that the standard curve was nearly perfectly linear and highly reproducible. Figure 2 illustrates chromatograms with 100 pg (370 fmol; Panel A) and 1 ng (3.7 pmol; Panel B) of adenosine in mobile phase. The signal-to-noise ratio was approximately 3 and 14 with 100 pg and 1 ng of adenosine, respectively.

**Step 2: Recovery of Adenosine and N²-Dimethylguanosine from Extraction, Filtration, and Sample Volume Reduction Procedures**

In five plasma samples percentage recoveries for adenosine and N²-dimethylguanosine through all steps of the assay, beginning with deproteinization and ending with volume reduction and reconstitution in mobile
Figure 1. Standard curve for adenosine in HPLC mobile phase. A standard curve was prepared by adding various amounts of adenosine to 100 ng of N2-dimethylguanosine (internal standard). The peak height ratio of adenosine to internal standard was plotted as a function of nanograms of adenosine per 100 ng of internal standard. The standard curve was repeated 4 weeks later using the same solutions. Equations indicate best-fit lines by linear regression.

Phase, were 44.1 ± 2.6% and 47.8 ± 2.4% (SEM), respectively. These data indicate that the losses of adenosine and N2-dimethylguanosine through the entire assay were quite similar.

Step 3: Relationship Between Amount of Adenosine Added to Supernatant Fluid and Amount Detected by Plasma

As illustrated in Figure 3, the amount of adenosine detected by the assay was linearly related ($r = 0.998$, $p < 0.001$) to the amount of exogenous adenosine added to the plasma sample. Further, the slope of this linear relationship was 0.95, indicating a nearly one-to-one correspondence between added and detected adenosine.

Figure 2. Sensitivity of Isco micro-liquid chromatography system to adenosine (100 pg [A] and 1 ng [B]) dissolved in mobile phase. Detector sensitivity was 0.005 absorbance unit full scale.

Figure 3. Relationship between amount of adenosine added to plasma and amount of adenosine detected by the assay.

Step 4: Stability of Adenosine in Blood Sample Collected into Stopping Solution

A plasma sample was assayed in quadruplicate immediately after sample collection and following a 2-hour delay. The plasma adenosine levels were 92 and 88 pg/ml, respectively, indicating no significant change in adenosine levels after the blood sample was mixed with stopping solution.

Step 5: Coefficient of Variation

The coefficient of variation determined by analyzing six identical plasma samples was calculated to be 7.7% (mean ± SD, 234 ± 18 pg/ml).
Step 6: Adenosine in Sample Treated with Adenosine Deaminase

Figure 4 depicts chromatograms obtained from two plasma samples from the same animal. However, one sample (Panel B) was incubated with adenosine deaminase, whereas the other sample (Panel A) was not. Adenosine deaminase eliminated the adenosine peak, indicating that this peak was indeed adenosine. Figure 4 also illustrates the baseline separation of adenosine and internal standard from all other components in the sample.

Step 7: Effect of Adenosine Infusion on Plasma Adenosine Level

Adenosine was infused intravenously into three anesthetized rats, and the relationship between the rate of adenosine infusion and plasma adenosine concentration was determined. As illustrated in Figure 5, infusion of synthetic adenosine resulted in a linear increase in plasma adenosine level.

Step 8: Adenosine Plasma Level in Normal Rats

The mean plasma adenosine level in 10 rats was 119 (0.44 $\mu$M) ± 28 (SD) ng/ml.

Renal Venous Plasma Levels of Adenosine in Sham-Operated and 2K1C Goldblatt Hypertensive Rats

Figure 6 depicts the values for renal venous plasma levels of adenosine in sham-operated and 2K1C rats. Plasma adenosine levels were elevated approximately fivefold to sixfold in both the left and right renal veins of 2K1C rats as compared with sham-operated controls ($p<0.001$). Figure 7 illustrates several chromatograms obtained from these experiments.

Discussion

This report describes a microassay for adenosine in rat plasma. The assay is accurate, simple, and inexpensive and permits the quantitation of adenosine in 75 $\mu$l of rat plasma. The improved sensitivity of this assay, relative to other reported plasma adenosine assays, allows for repeated measurement of plasma adenosine in the rat without inducing unacceptable blood loss.

The enhanced sensitivity of the adenosine microassay described in this report was obtained by using the full potential of microbore HPLC. Although microbore HPLC columns have been available for some time, much of the theoretical improvement in mass sensitivity has not been realized because of the large volumes in the HPLC injector, interconnecting tubing, and UV flow cell. Recently, HPLC systems with greatly reduced extracolumn volumes have become commercially available. The Isco microLC system, used to develop the assay in this report, is constructed to minimize extracolumn volume by eliminating interconnecting tubing and by using a low volume injector and UV flow cell. This physical arrangement reduces extracolumn volume to vanishingly small volumes and maximizes mass sensitivity. The signal-to-noise ratio is en-
enhanced further by providing solvent flow with a pulseless syringe pump, as opposed to a reciprocating pump, and by employing a microbore column with approximately 11,000 theoretical plates. The increased column efficiency also provides baseline separation of contaminating substances from adenosine and the internal standard.

As illustrated in Figure 1, the standard curve for adenosine in mobile phase using the Isco microLC system was linear. In addition, the standard curve for a particular set of standards did not change appreciably over 4 weeks, demonstrating a high degree of reproducibility and suggesting that frequent calibration with a standard curve is unnecessary.

The accuracy of the adenosine microassay is indicated by the experiment illustrated in Figure 3. As shown, a one-to-one correspondence between the amount of adenosine added to plasma and the amount detected by the assay was observed. Further, the specificity of the assay was demonstrated by the disappearance of adenosine from samples treated with adenosine deaminase (see Figure 4).

To determine whether the assay can detect increases in plasma adenosine levels, adenosine was infused in-
travenously into anesthetized rats. As shown in Figure 5, infusing exogenous adenosine caused a linear dose-dependent increase in plasma adenosine levels. This experiment demonstrates that if endogenous adenosine is released into the blood, it will be detected by the assay.

The arterial plasma level of adenosine in normal rats determined by the adenosine microassay was 119 ng/ml. This value corresponds closely with the normal levels reported for humans (135 ng/ml and 76 ng/ml), rabbits (267 ng/ml), dogs (70 ng/ml), and rats (80 ng/ml). However, von Borstel et al. recently reported that the normal plasma adenosine level in the rat is 800 ng/ml. The reason for this discrepancy is not clear, but it may reflect differences in experimental conditions (e.g., source of rats).

One practical point must be mentioned. The Isco microbore columns are protected with a 0.45-μm filter built into the column frit. Despite sample filtration, the column pressure gradually increases from a normal level of about 450 psi (at 20 μl/min) as the 0.45-μm column filter becomes increasingly plugged with particulates from the plasma samples. This problem can be reduced by placing a 2-μm filter (Part 720225; outside diameter, 1/16 in.; thickness, 1/32 in.; Alltech, Deerfield, IL, USA) directly behind the injection port of the Valco microinjector valve. This provides sample filtration immediately before the sample reaches the column. With this precaution, 25 to 30 plasma samples generally can be processed before the column pressure begins to increase and retention times become erratic. Once this happens, the 2-μm filter in the injector and the 0.45-μm filter in the column should be cleaned by sonication for 10 minutes each with nitric acid (6 M), water, and methanol.

Samples can be injected every 24 minutes without risk of compounds retained from a previous injection eluting during the chromatogram of a subsequent sample. After elution of the internal standard, at most four other unknown peaks will appear in the chromatogram. This phenomenon can be seen most clearly in Figure 4 (B). The last peak has a retention time of about 23 minutes, and once this unknown compound is eluted, no other substances can be detected in the chromatogram.

The primary motivation for developing this assay was to determine the effect of unilateral renal artery clamping on renal venous adenosine levels. Recently, we found that caffeine severely worsens the development of 2K1C Goldblatt hypertension in rats. The elevation of arterial blood pressure by caffeine was associated with a sevenfold increase in plasma renin activity. To explain this phenomenon, we hypothesized that renal adenosine levels may rise during renovascular hypertension and act to restrain the renin release response to renal artery ischemia. Since caffeine is an adenosine antagonist, if this hypothesis is true, then caffeine would elevate renin release by preventing the normal restraining function of adenosine. Consequently, caffeine would elevate plasma renin activity and, therefore, arterial blood pressure.

This scenario assumes that adenosine levels are elevated in renovascular hypertension and that adenosine attenuates the renin release response to renal artery ischemia. We recently discovered that adenosine does indeed restrict the renin release response to renal artery hypertension (unpublished observation 1986), and in the present study we found that renal levels of adenosine increase in renovascular hypertension. Therefore, so far our data support the hypothesis that endogenous adenosine functions to restrain the renin release response to renal artery stenosis. However, additional studies are needed to further clarify the role of adenosine in renin release in renovascular hypertension. Also, additional work is necessary to elucidate the source of renal venous adenosine in renovascular hypertension and the time course of the adenosine response.

In summary, this report describes and validates a microassay for adenosine in rat plasma. The assay is sensitive enough to measure adenosine levels in 75 μl of plasma from normal rats, thus permitting repeated determinations in an individual rat. Further, we report that renal venous adenosine levels are substantially elevated 1 week after unilateral renal artery stenosis in rats.

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